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A Derepression System Based on the *Bacillus subtilis* Sporulation Pathway Offers Dynamic Control of Heterologous Gene Expression▽

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By rewiring the sporulation gene-regulatory network of *Bacillus subtilis*, we generated a novel expression system relying on derepression. The gene of interest is placed under the control of the *abrB* promoter, which is active only when Spo0A is absent, and Spo0A is controlled via an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter.

We designed a novel system to express and secrete (heterologous) proteins in *Bacillus subtilis*. Instead of using an inducer to activate protein production, we used a derepression system. This allows growth of the host to high cell densities in the presence of the inducer. Upon removal of the inducer, high expression levels are obtained. To generate such a system, we made use of the well-studied sporulation phosphorelay of *B. subtilis*.

Controlled derepression of *abrB* by relief of Spo0A. As an adaptive ability in response to starvation, *B. subtilis* is able to form highly resistant endospores (13). The process of sporulation is governed by a multicomponent phosphorelay (11). Multiple environmental and physiological signals are fed into this system, and under appropriate conditions this leads to phosphorylation of Spo0A (Spo0A~P), the key sporulation transcription factor (1). A major role of Spo0A~P is to repress *abrB* expression (9, 10).

In the absence of Spo0A~P, *abrB* gene expression is constitutively high (12). To test whether we could use this feature of the sporulation pathway to construct a derepression system to express heterologous proteins in *B. subtilis*, we cloned the gene encoding green fluorescent protein (GFP) behind the *abrB* promoter (Fig. 1). In the resulting strain (17), cells highly express GFP during exponential growth, and fluorescence is reduced upon entry into the stationary growth phase (data not shown). Ireton et al. have shown that the *abrB* promoter is repressed by artificial induction of Spo0A-Sad67 (herein called Spo0A*), a constitutively active variant of Spo0A (3). Knowing this, we introduced the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible Spo0A* construct into our P_{abrB}-*gfp* strain, named A-gfp. All bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown and transformed using standard techniques.

Flow cytometric measurement of GFP fluorescence. Cells were grown overnight in TY medium (16) containing 100 μM IPTG and diluted 30-fold to an optical density at 600 nm (OD_{600}) of 0.05. At an OD_{600} of ~1.0, cells were diluted 10-fold in minimal medium, and a 1-ml suspension (without glass beads) was treated with a Mini-Bead-Beater-8 (Biospec Products) for 1 min at maximal speed to separate cell chains into individual cells. Two hours later (end of log phase), another sample was measured. GFP fluorescence was measured using a Coulter Epics XL-MCL flow cytometer (Beckman Coulter). The average fluorescence of 20,000 cells was determined using WinMDI 2.8 (http://facs.scripps.edu/software.html) and plotted against IPTG concentrations (Fig. 2). As shown in Fig. 2, GFP expression under the control of P_{abrB} is high without inducer but is strongly reduced upon increases in levels of Spo0A*. When the native *spo0A* gene is deleted, GFP expression is further increased. The maximum concentration of GFP in this strain was quantified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) relative to bovine serum albumin standards. Dry-matter concentrations of biomass were calculated using a predetermined correlation factor of 0.33 g (dry weight) of cells per OD_{600} unit (19). The concentration of GFP with an average fluorescence per cell of 550 arbitrary units was 16.7 mg GFP/g (dry weight). Interestingly, under inducing conditions (in the presence of Spo0A*), P_{abrB} is more tightly repressed in the *spo0A* (Δ*spo0A*) mutant. In the wild-type strain, the *abrB* promoter is still leaky and shows optimal derepression of *abrB* when Spo0A* is induced with 50 μM IPTG.

Secretion of *Clostridium perfringens* β-toxoid. To examine whether the system also enables the controlled expression of a secreted heterologous protein, we placed the gene encoding the *C. perfringens* β-toxin (*cpb*) behind the *abrB* promoter and combined this construct with the strain carrying the *spo0A* mutation (Δ*spo0A*) and the inducible Spo0A*.

The full coding sequence for β-toxin (*cpb*) was amplified by PCR with plasmid pXB10 as a template. *C. perfringens* β-toxin is a secreted protein with a Sec-type signal sequence and is an important component in animal vaccines against *C. perfringens* types B and C (7).

To visualize β-toxin secretion, total medium proteins were 10× concentrated by trichloroacetic acid precipitation and sep-

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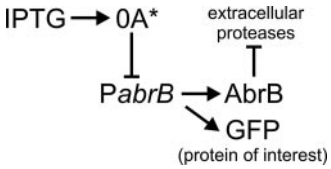

FIG. 1. Sporulation-based derepression system. A simplified schematic representation of the regulatory network used in this study is shown. Perpendicular symbols and arrows represent negative and positive actions, respectively. 0A* represents P_{spac}-*spo0A-sad67*. High levels of IPTG induce Spo0A*, which represses P_{abrB}. AbrB and the protein of interest are not produced, and there is no repression of the extracellular proteases. If IPTG is absent, Spo0A* is not produced and P_{abrB} is active, yielding high levels of AbrB and the protein of interest, while AbrB concomitantly represses the extracellular proteases.

arated by SDS-PAGE as described previously (14). β-Toxin was detected using Western blotting as described previously (7). As shown in Fig. 3A, at high IPTG induction levels, no β-toxin could be detected. Upon derepression from Spo0A*, β-toxin accumulated in the growth medium. These results show the versatility of the derepression system and demonstrate that (heterologous) gene expression can be accurately controlled.

To examine whether the described derepression system can be activated in cultures with a high cellular density, which is required when producing toxic products, we grew strains A-β (P_{abrB}-*cpb*) and A-β/0A*/Δ0A (P_{abrB}-*cpb* P_{spac}-*spo0A** Δ*spo0A*) to dense cultures in TY medium containing 250 μM IPTG (full repression). Next, cells were spun down, washed once, and resuspended in fresh TY medium without IPTG. Medium fractions were collected at timely intervals and assayed for β-toxin secretion. As shown in Fig. 3B, within 20 min after resuspension, P_{abrB} was derepressed in A-β/0A*/Δ0A and β-toxin could be detected in the growth medium. β-Toxin continued to accumulate in the medium up to 2 h after derepression in this dense culture, and we were able to recover β-toxin until 5.5 h after suspension. In the presence of a functional *spo0A* gene, however, secreted protein could not be observed after 3.5 h.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties	Reference
Strains		
E. coli MC1061	F⁻ *araD139* Δ(*ara-leu*)*7696* Δ(*lac*)X74 *galU galK hsdR2 mcrA mcrB1 rspL*	18
B. subtilis		
168	*trpC2*	4
SWV215	*spo0A*::Km	20
0A* (*sad67*::Tc)	*amyE*::P_{spac}-*spo0A-sad67* Tc^r	16
A-gfp (AbrB-GFP)	P_{abrB}-*gfp* Cm^r	17
0A*/A-gfp	*amyE*::P_{spac}-*spo0A-sad67* P_{abrB}-*gfp* Cm^r Tc^r	This study
Δ0A/A-gfp	*spo0A*::Km P_{abrB}-*gfp* Cm^r Km^r	This study
0A*/Δ0A/A-gfp	*amyE*::P_{spac}-*spo0A-sad67* *spo0A*::Km P_{abrB}-*gfp* Cm^r Tc^r Km^r	This study
A-β	P_{abrB}-*cpb* Cm^r	This study
0A*/A-β	*amyE*::P_{spac}-*spo0A-sad67* P_{abrB}-*cpb* Cm^r Tc^r	This study
Δ0A/A-β	*spo0A*::Km P_{abrB}-*cpb* Cm^r Km^r	This study
0A*/Δ0A/A-β	*amyE*::P_{spac}-*spo0A-sad67* *spo0A*::Km P_{abrB}-*cpb* Cm^r Tc^r Km^r	This study
Plasmids		
pSG1151	*bla cat gfp*	5
pXB10	pUB110 containing β-toxin coding region	15
pP_{abrB}-*cpb*	*bla cat* P_{abrB}-*cpb*	This study

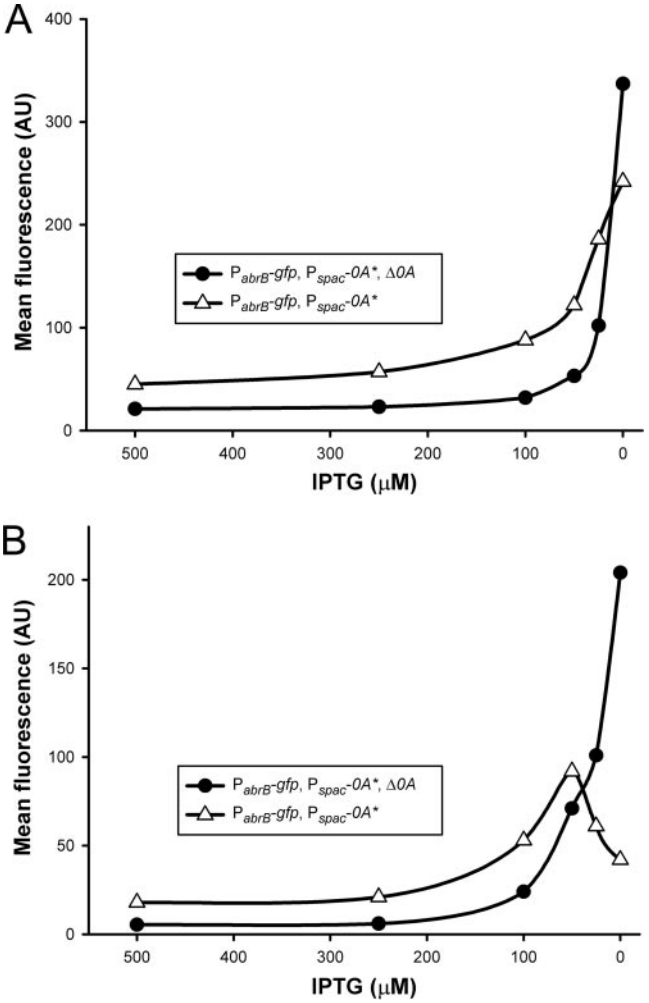

FIG. 2. Controlled activation of P_{abrB}-*gfp* via derepression. Expression of Spo0A* was induced with various concentrations of IPTG. P_{abrB} expression was measured during mid-exponential growth (A) and late exponential growth (B) using a P_{abrB}-*gfp* fusion. Expression of P_{abrB}-*gfp* in the presence and absence of endogenous *spo0A* was measured (strains 0A*/A-gfp and 0A*/Δ0A/A-gfp). The units for GFP fluorescence are arbitrary units (AU) and are identical in panels A and B.

***abrB* derepression results in reduced extracellular proteolytic activity.** Our results show that secreted β-toxin is more actively degraded in the presence of a functional *spo0A* gene. Many of the known extracellular proteases are under either the direct or indirect control of Spo0A (6). To examine whether the increased degradation of β-toxin is related to increased extracellular proteolytic activity, we assayed the growth medium for proteolytic activity. Total protease activity was measured using the Roche resorufin-labeled universal protease substrate as described by the manufacturer. Direct optical readout of the OD_{574} was plotted. As depicted in Fig. 3C, only a minor proteolytic activity was measured in the Δ*spo0A* strain. In the presence of *spo0A*, strong protease activity was observed

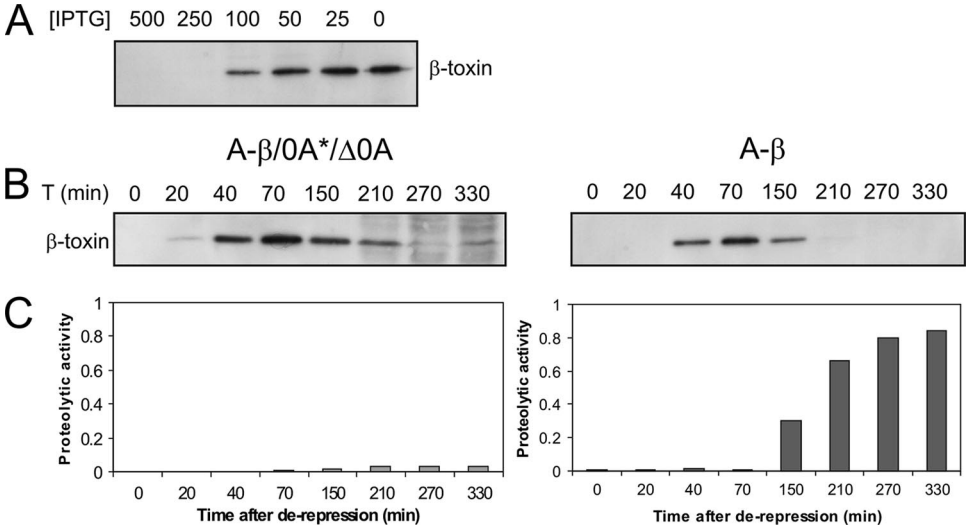

FIG. 3. Controlled secretion of β-toxin via derepression. *abrB* derepression results in reduced extracellular proteolytic activity. (A) Detection of 35-kDa β-toxin secreted by strain 0A*/Δ0A/A-β (P_{abrB}-*cpb* P_{spac}-*spo0A** Δ*spo0A*). (B) Detection of secreted 35-kDa β-toxin by strains 0A*/Δ0A/A-β (P_{abrB}-*cpb* P_{spac}-*spo0A** Δ*spo0A*) and A-β (P_{abrB}-*cpb*). Time (T) zero is directly after resuspension in fresh medium. The increased background in later time points for 0A*/Δ0A/A-β is caused by cross-reaction of the β-toxin antibody with some intracellular proteins released into the medium by cell lysis. (C) Extracellular proteolytic activity of the cultures depicted in panel B.

starting after 150 min of resuspension in fresh medium, when cells enter stationary growth phase (data not shown).

Induction and deletion of *spo0A*. The use of sporulation-deficient mutants (such as the Δ*spo0A* strain) is common practice for large-scale high-density fermentation processes, mainly because it prevents the formation of spores, which are hard to remove from the growth system (8). Strain stability is not an issue for these mutants, since sporulation is purely an adaptive phenotype and not essential. The addition of IPTG in the case of our Spo0A* strain led to induction of Spo0A, and therefore undesirable sporulation could occur more rapidly. However, we did not observe sporulation under our culture conditions. The process of sporulation is tightly controlled, and when only *spo0A* is induced during logarithmic growth, not all essential components for sporulation are expressed, preventing premature sporulation of the culture (2).

Concluding remarks. In this paper we show how a naturally occurring gene-regulatory network can be adapted to serve as a tailored expression system. An additional benefit of using this endogenous pathway is that the system is based on self-cloning via homologous recombination, keeping the introduced foreign DNA to a minimum. Furthermore, upon activation of the *abrB* promoter, extracellular proteolytic activity is reduced.

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